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Chemical properties and antioxidant activity of sweetened red ginger extract fermented with kombucha culture

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ABSTRACT

Kombucha is a renowned fermented drink for its diverse health benefits. This research aims to evaluate sweetened red ginger extract as an alternative substrate in making kombucha by observing the chemical properties (pH, total acid, total sugar, total polyphenol, chemical compound profile using LC-MS/MS) and the antioxidant activity. Three variables were varied, i.e., red ginger concentration (1, 5, and 10%), kombucha culture concentration (10 and 20%), and fermentation time (0, 6, and 12 days). The total sugar and pH declined during fermentation while the titratable acidity, polyphenol, and antioxidant activity increased. The kombucha prepared with 20% culture concentration resulted in a greater reduction of pH and increase of titratable acidity, total polyphenol, and antioxidant activity than the one prepared with 10% culture concentration. The red ginger kombucha prepared with 10% ginger and 20% culture in 12 days displayed the highest antioxidant activity. It revealed ten active compounds under the LC-MS/MS investigation, i.e., 3',4',5',5,7,8-hexamethoxy flavone, 6-gingerol, evodin, isosakuranetin-7-rutinoside, methyl ophiopogonanone A, naririutin, neohesperidin, ononin, sinensetin, and shogaol. This research shows that red ginger extract fermentation using kombucha culture can be an alternative technology to produce red ginger-based functional drinks with healthy organic acids, healthy polyphenols, and antioxidant activity.

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INTRODUCTION

Nowadays, the demand for functional foods is unquestionably rising as people are more alarmed about being healthy. Kombucha is a renowned fermented drink loaded with various health benefits. It is easily made by adding a symbiotic consortium of acetic acid bacteria and yeast into sweetened tea. The kombucha has unique characteristics of a slightly sweet and sour taste, imparting a refreshing sensation (Ayed et al. 2017).

Kombucha microbial biodiversity is dependent on local climate, geographic condition, local species of bacteria and yeast, and inoculum source. Acetic acid bacteria and osmophilic yeasts generally inhabit kombucha. Acetic acid bacteria include *Acetobacter* (e.g., *Acetobacter xylinum*, *Acetobacter xylinoides*, *Acetobacter aceti*, and *Acetobacter pausterianus*) and *Gluconobacter* (e.g., *Bacterium gluconicum*). *Acetobacter xylinum* is well known for its fermenting ability and forms a floating cellulose layer on the air-liquid interface of kombucha. Yeasts that are commonly present in kombucha include *Candida stellimalicola*, *Candida tropicalis*, *Lachancea thermotolerans*, *Lachancea fermentati*, *Eremothecium cymbalariae*, *Kluyveromyces marxianus*, *Pichia mexicana*, *Dekkera bruxellensis*, *Dekkera anomala*, *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Zygosaccharomyces bailii*, and *Zygotorulaspora florentina* (Jayabalan et al. 2014; Villarreal-Soto et al. 2019; Watawana et al. 2015). Lactic acid bacteria (BAL) are also commonly found in kombucha and considered probiotics, e.g., *Lactobacillus kefiranofaciens*, *Lactobacillus nagelli*, *Lactobacillus satsumensis*, and *Lactococcus* sp. (Bogdan et al. 2018; Marsh et al. 2014).

During fermentation, yeast and bacteria work symbiotically. Yeasts hydrolyze sucrose into fructose and glucose and yield ethanol through glycolysis. Acetic acid bacteria consume glucose to produce gluconic acid and ethanol, furthermore converting ethanol to acetic acid (Jayabalan et al. 2014). The resultant ethanol and acetic acid stimulate the growth of acetic acid bacteria and yeast, respectively (Liu et al. 1996).

Kombucha brew has numerous bioactive compounds which contribute to its health benefits. Some compounds are present in the initial substrate, while others are produced during fermentation. Organic acids (e.g., acetic, gluconic, glucuronic, lactic, malic, citric, and tartaric acids), water-soluble vitamins (B1, B2, B6, B12, and C), amino acids, minerals, polyphenols, and enzymes are commonly found in kombucha. The benefits of kombucha include improving the immune system, harmful detoxifying substances, lowering blood pressure, treating gastritis and cholesterol, and exhibiting antioxidant, antibacterial, anticancer, and antidiabetic activities (Jayabalan et al. 2014).

Besides tea, kombucha can be prepared with other substrates, e.g., red grape (Ayed et al. 2017), pomegranate (Yavari et al. 2018), and snake fruit (Zubaidah et al. 2018) juices. Melanie et al. (2017) have developed kombucha from spinach. Spinach kombucha was produced using kombucha inoculum previously cultured in spinach substrate (spinach extract supplemented with 10% (w/v) sucrose was fermented using 10% tea kombucha).

Many studies report that the composition and concentration of metabolites in kombucha production are affected by various factors, such as fermentation time (Chen and Liu 2000), culture concentration (Iličić et al. 2017; Melanie et al. 2017), also the nature and composition of the substrates medium (Marsh et al. 2014). Melanie et al. (2017) show that fermentation of spinach extract in 0-14 days affected the chemical composition of spinach kombuchas, such as total polyphenols, soluble protein, and acid. Iliči et al. (2017) developed kombucha milk with a 10-15% culture concentration. They reported that the culture concentration affected the total sugar, acid, and ethanol content of kombucha milk. Pebiningrum et al. 2018 investigated kombucha from three types of ginger (emprit ginger, elephant ginger, and red ginger) using variations in honey concentrations (10, 15, and 20%). In this study, different substrate compositions affected ginger kombucha's chemical and antioxidant properties.

Red ginger is an interesting candidate for kombucha ingredients due to its unique taste and numerous health activities, e.g., antidiabetic, antioxidant, ACE-2 enzyme inhibitor, anticarcinogenic and antimutagenic (Suciyati and Adnyana 2017; Syafitri et al. 2018). Red ginger contains volatile components such as terpenoids and non-volatile components, including flavonoids and polyphenols (6-gingerol and its derivatives) (Stoilova et al. 2007). The strong antioxidant properties of ginger are linked to its

bioactive compounds, e.g., 6-shogaol, 6-gingerol, and oleoresin (Mao et al. 2019). To the best of our knowledge, the utilization of ginger is limited to certain products, e.g., candies, syrups, extracts, and instant powder drinks (Anjani et al., 2021).

The utilization of red ginger extract as an alternative substrate for kombucha preparation is appealing to diversify the incorporation of ginger in functional food. This research aimed to evaluate the potential of red ginger extract for kombucha preparation, particularly through chemical (i.e., pH, titratable acid, total sugar, total polyphenol), LC-MS/MS, and antioxidant investigation. The effect of red ginger concentration, kombucha culture concentration, and fermentation time on ginger kombucha's chemical content was also investigated in this research.

MATERIAL & METHODS

Red ginger (*Zingiber officinale* var rubrum) and sucrose were purchased from a traditional market. Bottled water was used in the preparation of kombucha. Kombucha inoculum was bought from kombucha brewer "Indokombucha," who traditionally grows the culture in sweetened tea. It comprised the cellulose layer and the kombucha liquid.

Chemicals used for analysis, i.e., 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (ABTS), potassium persulfate, trolox, gallic acid, folin ciocalteau, sulfuric acid, phenol, sodium carbonate, natrium hydroxide, glucose, formic acid, and acetonitrile from Merck.

Several instruments used were balanced (Mettler Toledo, Switzerland), analytical balance (Kern ABJ 320-420, Balingen), blender (Philips, Netherland), centrifuge (Eppendorf AG, USA), vortex (Thermolyne Maximix Plus TM., USA), spectrophotometer (Agilent Technologies Cary 60 UV-Vis, USA), pH meter (Eutech Instruments pH 700, Singapore), and LC-MS/MS Xevo G2-XSQTop (Waters, USA).

Preparation of Kombucha Inoculum with Red Ginger Extract as the Substrate

Red gingers were washed to remove dirt, and the red ginger juice was prepared at a ginger concentration of 1% w/v. The juice was boiled for 10 minutes, added with 10% sucrose (w/v), and finally filtered with an 80-mesh filter to yield a sweetened red ginger extract. The extract was left to cool down (27-30ºC) before being transferred into a sterilized glass jar and inoculated with a 20% kombucha starter (v/v). The container was secured with a clean fine cloth and left to ferment for 30 days to produce the inoculum for the subsequent brewing.

The production of red ginger kombucha

Red ginger juices at higher concentrations (1%, 5%, and 10% w/v) were prepared for kombucha brewing. The juices were boiled for 10 minutes, added with 10% sucrose w/v, and filtered with an 80-mesh filter to yield sweetened red ginger extracts. The fermentation was done using two different inoculum concentrations (10% and 20% v/v) for 0, 6, and 12 days.

Determination of pH

The pH of the samples was tested with a digital pH meter (Eutech Instruments pH 700, Singapore). This analysis was carried out in duplicate.

Determination of Titrable Acidity

The titratable acidity was measured by titration to the sample with 0.1 M NaOH, and the result was expressed in acetic acid percentage (AOAC, 1984). The formula for calculating acid content:

$$Acid\ content\ \% = \frac{V1\ x\ N\ x\ B}{V2\ x\ 1000}\ x\ 100\%$$

V1 = NaOH volume (mL)
V2 = Sample volume (mL)
N = NaOH Normality (0.1 N)
B = Molecular weight of acetic acid (60)

Determination of Total Sugar

The total sugar was determined by following the phenol-sulfuric acid assay (Dubois et al. 1956). A total of 50 µL of the sample was added with 750 µL of distilled water, 40 µL of 5% phenol, and 2 mL of concentrated H_2SO_4, then shaken and incubated in a dark room for 30 minutes. Samples were duplicated, and absorbance was measured on a UV-Vis spectrophotometer with a wavelength of 490 nm. Total sugar content was calculated by plotting the absorbance value obtained on the glucose standard that has been made.

Determination of Polyphenol Content

Total polyphenol content was measured using the Folin-Ciocalteu method with a gallic acid standard curve (Singleton et al. 1999). A total of 100 µL of the sample was added with 250 µL of Folin Ciocalteu phenol reagent (The ratio

between Folin Ciocalteu reagent and water is 1:1) and incubated for 8 minutes. Next, the sample was added with 750 mL of 10% Na_2CO_3 solution and set for 2 hours. The sample was added with 3.9 mL of distilled water, shaken, and read the absorbance using a UV-Vis spectrophotometer at a wavelength of 740 nm. The analysis was performed in duplicate, and the total phenolic contents (TPC) were calculated using the standard gallic acid curve, presented in ppm units.

Antioxidant (ABTS assay)

This study followed the ABTS procedure from (Delgado-Andrade et al. 2005) with ABTS solution at 7 mM and potassium persulfate solution at 140 mM. The radical ABTS solution was prepared by mixing 20 mL of ABTS solution with 352 µL of 140 mM $K_2S_2O_8$ solution and leaving the mixture in a dark room for 18 hours. Later, 1 mL of radical ABTS solution was diluted with 5 mL of H_2O to achieve the UV-Vis spectrophotometer absorbance of 0.75 at 734 nm. As much as 25 µL of the sample was mixed with 1 mL of radical ABTS solution. The mixture was incubated for 6 minutes before spectrophotometric assay at 734 nm. The control was prepared similarly with Trolox instead of the kombucha sample. The result was expressed in the percent of inhibition (%). This assay was performed in duplicate.

$$\%\ Inhibition = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}}\ x\ 100\%$$

Abs blank = absorbance of the blank
Abs sample = absorbance of the sample

LC-MS/MS Analysis of Red Ginger Kombucha

The sample with the highest antioxidant activity was extracted with ethyl acetate before LC-MS/MS analysis. LC-MS/MS analysis was performed using a Xevo, G2-XS QTof (Waters MS Technologies, USA), equipped with Waters Acquity Ultra Performance Liquid Chromatography (UPLC).

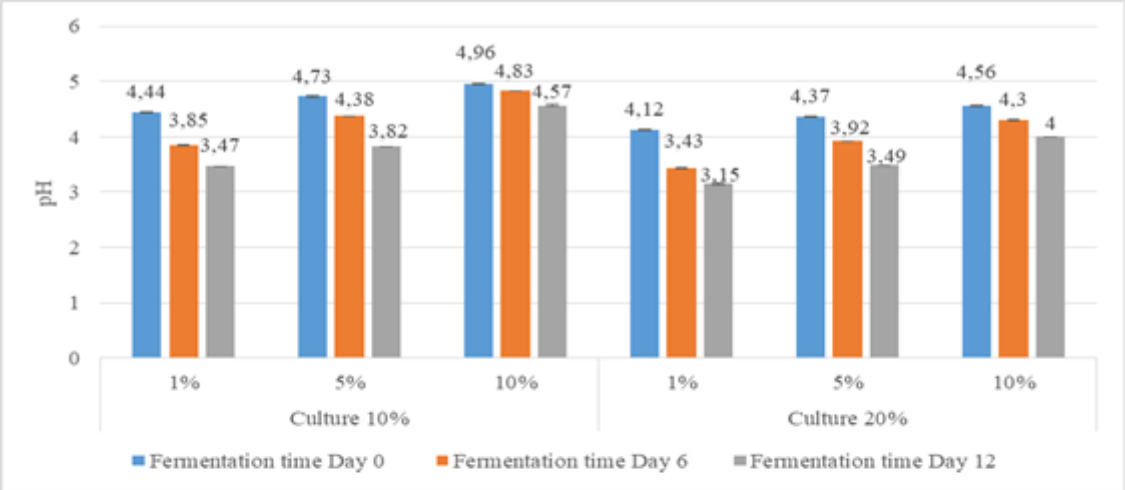
The condition of the mass spectrometry was as follows: capillary voltage 2 kV; source temperature 120°C; cone gas flow 30 kV; desolvation temperature 500°C; and desolvation gas flow 1000 L/h. The mass range detection started at 50 m/z and ended at 1200 m/z with an ESI ionization positive mode. ACQUITY UPLC BEH C8 column 100 mm x 2.1 mm, 1.7 µm was used in the chromatographic separation system with the column temperature set at 40°C. The moving phase consisted of solvent A (H_2O + 0.1 % Formic Acid (FA)) and solvent B (acetonitrile + 0,1 FA) with gradient elution (ratio of A to B) from 95:5 to 5:95. The flow rate was set at 0.3 mL/min. The volume of sample injection was 0.5 µL. UNIFI was employed for data acquisition and processing.

RESULT AND DISCUSSION

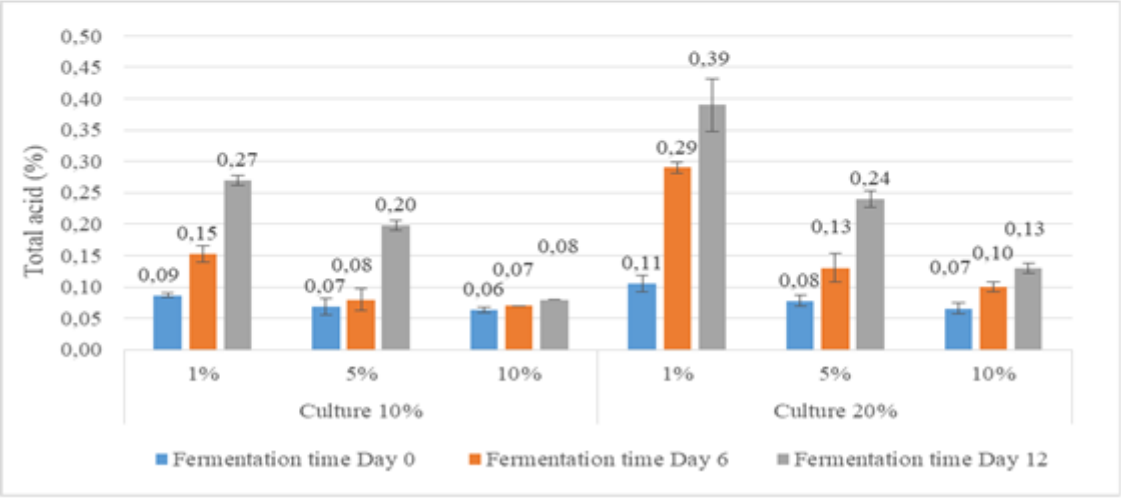
pH and Titratable Acidity of Red Ginger Kombucha

All samples displayed reduced pH and increased total acid as the fermentation time continued (Figure 1). A similar result was achieved by Ayed et al. (2017) and Zubaidah et al. (2018). Ayed et al. (2017) recorded a pH reduction from 3.95 to 2.91 and a total acid increase from 25.9 to 104.2 meq/L in their experiment with 12-day fermentation of 3% kombucha cellulose and 10% kombucha liquid. Zubaidah et al. (2018) fermented Suwaru snake fruit juice (fruit: water = 1:1) with 10% sugar and 10% starter for 14 days and resulting in pH reduction from 3.91 to 3.22 and total acid increase from 0.57% to 1.64%. In addition, The accumulation of organic acids in red ginger kombucha is responsible for pH reduction and total acid increase (Ayed et al. 2017). The decrease of pH in kombucha brewing is reported to be beneficial for preventing chemical degradation of polyphenol and maintaining the color of kombucha liquid (Ulusoy and Tamer 2019).

At the same red ginger concentration and fermentation time, the kombucha sample prepared with 20% culture resulted in a lower pH than the one prepared with 10% culture. Consequently, the total acid of samples with 20% culture was higher than those of the counterpart samples with 10% culture. It made sense because a higher initial culture concentration provided more microbes for kombucha brewing, yielding more organic acids. The initial culture also contained organic acids (as indicated by pH 3.46), thus affecting the pH and the total acid of the resultant kombucha. Iličić et al. (2017) also observed that kombucha prepared with 15% starter displayed a greater acid number compared to the counterpart kombucha prepared with 10% starter.



(a)



(b)

Figure 1 The changes of a) pH and b) total acid of the samples

When the red ginger concentration increased at both fixed initial culture concentration and fermentation time, we observed a milder pH drop and total acid rise in our kombucha samples. We proposed that the antimicrobial property of red ginger was the motive since Poelongan (2011) reported antibacterial activities against *Saccharomyces aureus, Saccharomyces epidermis*, and *Saccharomyces agalactiae* while Pundir et al. (2010) reported the antifungal properties of red ginger extract.

Total Sugar of Red Ginger Kombucha

The total sugar and the fermentation time of the red ginger kombucha were inversely proportional (Figure 2), implying that sucrose and other sugars in red ginger kombucha were consumed by the symbiotic colony of bacteria and yeasts. Sucrose is a traditional carbon source in the brewing of kombucha. Abuduaibifu and Tamer (2019) and Pratiwi et al. (2012) also reported a decrease in sugar during fermentation.

We proposed that the total sugar of red ginger kombucha contributed to the initial total sugar content in the sweetened red ginger extracts and the break down of complex sugars to simple sugars during fermentation. The sweetened red ginger extracts with a higher red ginger concentration displayed a greater total sugar even though the added sucrose remained the same. Our extracts prepared with 1%, 5%, and 10% of red ginger (w/v) and 10% of sucrose (w/v) showed 271.2, 286.3 and 292.8 mg/mL of total sugar, respectively. This result was anticipated since

gingers contain 50-70% carbohydrates (dry basis) (Grzanna et al. 2005).

As more carbohydrates were available for kombucha brewing, the resultant kombucha with a higher red ginger concentration exhibited an increased sugar content. Pebiningrum et al. (2018) believed this was due the amylase enzyme released by *Saccharomyces cerevisiae*.

At the same red ginger concentration and fermentation time, kombucha samples with 20% culture showed a greater total sugar than the counterpart samples with 10% culture, presumably due to the sugar content of the inoculum liquid. Iličić et al. (2017) discovered that kombucha with a 3% evaporated culture yielded more glucose than kombucha with a 1.5% evaporated culture.

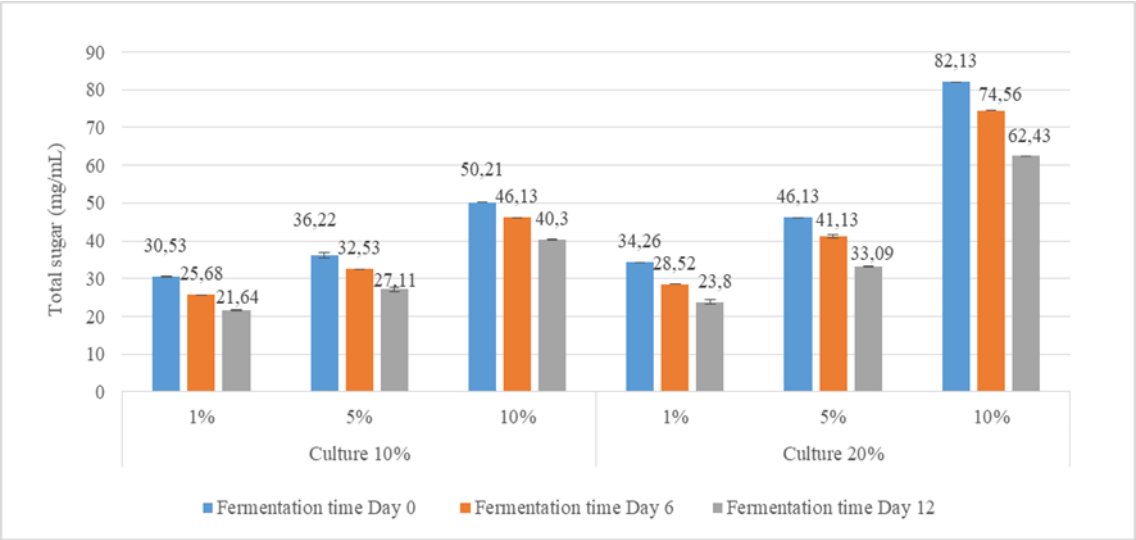


Figure 2 Total sugar content of red ginger kombucha

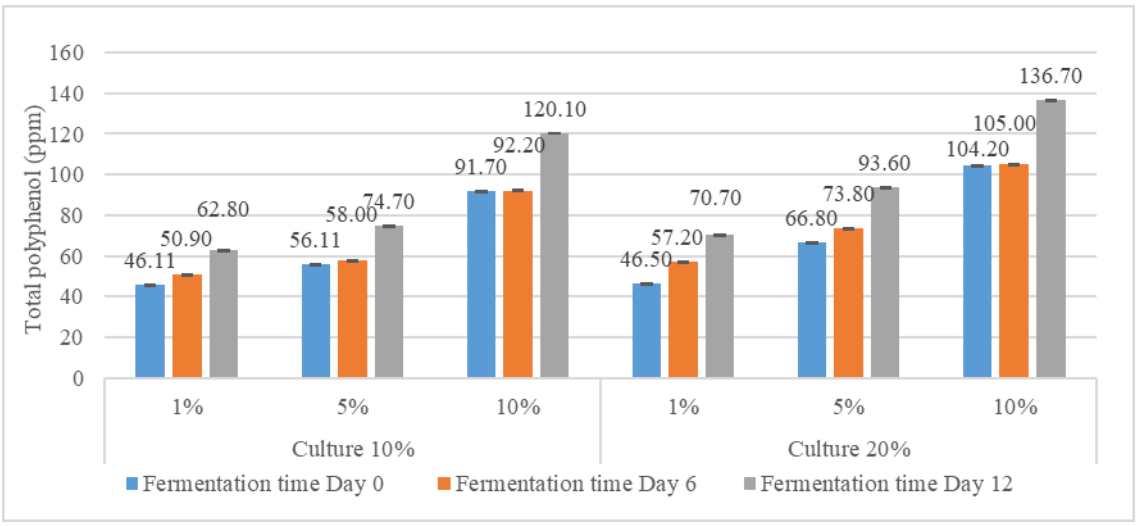


Figure 3 The effect of red ginger concentration, culture concentration and fermentation time to total polyphenol in red ginger kombucha

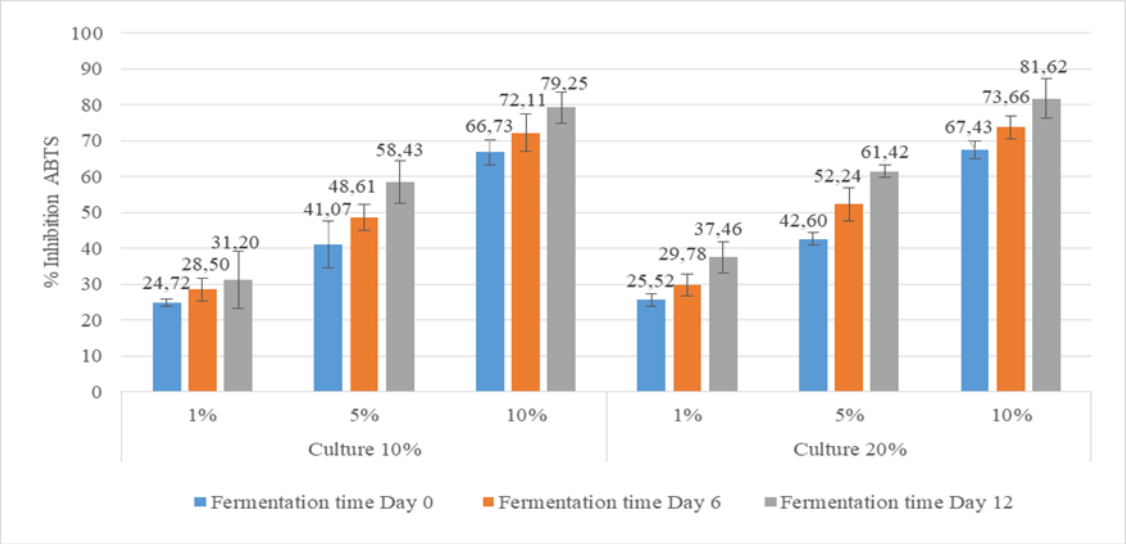


Figure 4 The influence of fermentation time, ginger concentration and culture concentration on antioxidant activity of red ginger kombucha

Table 1 LC-MS/MS profile of red ginger kombucha with the highest antioxidant activity

Component name	Observed m/z	Neutral mass (Da)	Observed RT (min)	Adducts	Detector counts	Formula
3',4',5',5,7,8-Hexame-thoxy flavone	403.1385	402.13147	8.01	+H, +Na	2030711	$C_{21}H_{22}O_8$
6-Gingerol	317.1728	294.18311	8.39	+Na	99794	$C_{17}H_{26}O_4$
Evodin	471.2014	470.19407	7.93	+H	327229	$C_{26}H_{30}O_8$
Isosakuranetin-7-rutinoside	595.2030	594.19486	6.03	+H, +Na	276526	$C_{28}H_{34}O_{14}$
Methyl ophiopogonanone A	343.1177	342.11034	7.60	+H	282597	$C_{19}H_{18}O_6$
Narirutin	581.1866	580.17921	4.99	+H, +Na	183129	$C_{27}H_{32}O_{14}$
Neohesperidin	611.1973	610.18977	5.20	+H, +Na	521242	$C_{28}H_{34}O_{15}$
Ononin	431.1337	430.12638	7.67	+H	307907	$C_{22}H_{22}O_9$
Sinensetin	373.1280	372.12090	7.49	+H, +Na	1967365	$C_{20}H_{20}O_7$
Shogaol	277.1801	276.17254	8.39	+H	534834	$C_{17}H_{24}O_3$

Total Polyphenol of Red Ginger Kombucha

The total polyphenol of the red ginger kombucha samples increased as culture concentration, red ginger concentration, or fermentation time went up (Figure 3). Some health benefits of kombucha are closely related to its polyphenol content. Ayed et al. (2017) reported that the polyphenol content of their fermented grape juice increased during 6-day fermentation. Chu and Chen (2006) also explained the increase of polyphenol content in their black tea kombucha during extended fermentation. Hydrolytic enzymes like cellulase, invertase, and amylase of kombucha culture can break down complex polyphenols into simple ones, thus improving the total polyphenol number during the assay (Zubaidah et al., 2019). Kombucha culture contains acetic acid bacteria (e.g., *Acetobacter* and *Gluconobacter*) and osmophilic yeasts. *Gluconobacter* enhanced polyphenol bioaccessibility (Vina et al. 2013). Yeasts such as *Candida tropicalis* were reported as polyphenol degrading agents (Ettayebi et al. 2003). Kombucha culture also contains some species of lactic acid bacteria, e.g., *Lactobacillus plantarum*, with the ability to break down polyphenols (Zhou et al. 2020). Red ginger is well known for polyphenols, e.g., gingerol and shogaol (Mošovská et al. 2015). Thus, our kombucha

samples which were prepared with a higher ginger concentration, displayed a higher total polyphenol. When the ginger concentration was fixed, a higher culture concentration also increased the total polyphenol of the resultant kombucha. We believed this was due to more polyphenol-degrading hydrolytic enzymes or organic acids in the culture. In addition, the kombucha inoculum contained polyphenols, which contributed to the increase of the total polyphenol of the resultant kombucha. In their study of broccoli kombucha, Melanie et al. (2017) displayed that a higher culture concentration led to a greater number of total polyphenol.

Antioxidant activity of Red Ginger Kombucha

The percent of ABTS inhibition of the red ginger kombucha samples increased as culture concentration, red ginger concentration, or fermentation time went up (Figure 4), with a similar trend to that of the total polyphenol (Figure 3).

Fermentation is known to have enhanced antioxidant activity due to the increased total polyphenol content due to microbial hydrolysis (Hur et al., 2014). As conjugated phenolic compounds were bioconverted to their free form, they showed more health benefits (Torino et al., 2013).

The antioxidant activity of ginger is strongly related to its polyphenol content since it contains phenolic components (6-gingerol and related compounds like shogaol) and terpenes terpene (α-zingiberene, β-bisabolene), which possess antioxidant properties (Contreras-López et al. 2020).

LC-MS/MS Analysis of Red Ginger Kombucha

LC-MS/MS analysis was conducted on samples with the highest antioxidant activity (red ginger kombucha prepared with 10% red ginger and 20% culture in 12-day-fermentation). It was extracted with ethyl acetate before LC-MS/MS analysis. Ten compounds were revealed in the extract (Table 1). Fresh ginger contains a notable amount of phenolic compounds and terpenes. The dominant phenolic compounds in fresh ginger are gingerol, shogaols, and paradols; gingerols (i.e. 6 ingerol, 8-gingerol, and 10-gingerol) act as the primary polyphenol (Mao et al. 2019). Gingerol can be converted to shogaol through either heat treatment or long period storage (Mao et al. 2019). Shogaols are reported to exhibit stronge ginger extracts for 10 minutes before brewing possibly induced the conversion of gingerol to shogaol. Furthermore, the increased organic acid during fermentation may promote the conversion of gingerol to shogaol (Ghasemzadeh et al., 2018). Other compounds in our samples besides gingerol and shogaol also exhibited bioactivities, e.g., anticancer, antioxidant, and antibacterial activities (Hilles et al. 2019) (Chen et al. 2021) (Li et al. 2018; Lin et al. 2015; Pan et al. 2021; Tomás-Navarro et al. 2014; Suryana 2010; Yao et al. 2018)

CONCLUSION

Red ginger concentration, kombucha culture concentration, and fermentation time influenced the pH, titratable acidity, total sugar, polyphenol, and antioxidant activity of red ginger kombucha. The fermentation resulted in the pH and total sugar decreasing while the values of titratable acidity, total polyphenol, and antioxidant activity improved. Fermentation of 10% red ginger using 20% culture for 12 days showed the highest antioxidant activity (81,62 % inhibition ABTS), with a pH value of 4, total acid of 0,13%, total sugar of 62,43 mg/mL, and polyphenol content of 136,7 ppm. LC-MS/MS analysis on the samples which have the highest antioxidant activity reveals the profile of its 10 chemical compounds is as follows: 3',4',5',5,7,8-Hexame-thoxy flavone, 6-Gingerol, Evodin, Isosakuranetin-7-rutinoside, Methyl ophiopogonanone A, Narirutin, Neohesperidin, Ononin, Sinensetin, and Shogaol.

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